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Temperature Effects on Hemocyanin Oxygen Binding in an Antarctic Cephalopod

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Abstract. The functional relevance of oxygen transport by hemocyanin of the Antarctic octopod *Megaleledone senoi* and of the eurythermal cuttlefish *Sepia officinalis* was analyzed by continuous and simultaneous recordings of changes in pH and hemocyanin oxygen saturation in whole blood at various temperatures. These data were compared to literature data on other temperate and cold-water cephalopods (octopods and giant squid).

In *S. officinalis*, the oxygen affinity of hemocyanin changed at $\Delta P_{50}/°C = 0.12$ kPa (pH 7.4) with increasing temperatures; this is similar to observations in temperate octopods. In *M. senoi*, thermal sensitivity was much smaller (<0.01 kPa, pH 7.2). Furthermore, *M. senoi* hemocyanin displayed one of the highest levels of oxygen affinity ($P_{50} < 1$ kPa, pH 7.6, 0 °C) found so far in cephalopods and a rather low cooperativity ($n_{50} = 1.4$ at 0 °C). The pH sensitivity of oxygen binding ($\Delta \log P_{50}/\Delta$ pH) increased with increasing temperature in both the cuttlefish and the Antarctic octopod. At low Po_2 (1.0 kPa) and pH (7.2), the presence of a large venous oxygen reserve (43% saturation) insensitive to pH reflects reduced pH sensitivity and high oxygen affinity in *M. senoi* hemocyanin at 0 °C. In *S. officinalis*, this reserve was 19% at pH 7.4, 20 °C, and 1.7 kPa O_2, a level still higher than in squid.

These findings suggest that the lower metabolic rate of octopods and cuttlefish compared to squid is reflected in less pH-dependent oxygen transport. Results of the hemocyanin analysis for the Antarctic octopod were similar to those reported for *Vampyroteuthis*—an extremely high oxygen affinity supporting a very low metabolic rate. In contrast to findings in cold-adapted giant squid, the minimized thermal sensitivity of oxygen transport in Antarctic octopods will reduce metabolic scope and thereby contribute to their stenothermality.

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Introduction

Cephalopods are found throughout the seas of the world, from warm tropical waters to polar oceans (Roper *et al.*, 1984). Representatives of this group, especially squids, usually display the highest metabolic rates among marine invertebrates, even higher than those of fishes of similar size and mode of life (Webber and O'Dor, 1985; O'Dor and Webber, 1986). Oxygen delivery *via* the blood is maximized to cover metabolic requirements (Pörtner, 1994). However, the capacity of their blood pigment, hemocyanin, for carrying oxygen is constrained by the low concentration of an extracellular pigment. This limitation is due to the unfavorable increase in colloidal osmotic pressure and blood viscosity at high pigment concentrations (Mangum, 1983, 1990). Although cephalopods, in accordance with their high rate of oxygen consumption, display the highest hemocyanin concentrations in the animal kingdom, the level of bound oxygen in squid (up to 3 mmol l^{-1}; Brix *et al.*, 1989) remains below the 4–5 mmol l^{-1} of active fishes (Urich, 1990). Therefore, the hearts of squids pump large volumes of blood (Wells *et al.*, 1988; Shadwick *et al.*, 1990) and the tissues extract most of the oxygen (Pörtner, 1994). Compared to that of squids, the oxygen-binding capacity of octopod blood is somewhat reduced, ranging between 0.6 and 1.6 mmol l^{-1}, depending on hemocyanin levels (Senozan *et al.*, 1988; Brix *et al.*, 1989).

In most cephalopods, cooperativity and temperature- and pH-dependent changes in affinity are the only means of modulating hemocyanin function and adjusting oxygen transport (*e.g.*, Brix *et al.*, 1989; Mangum, 1990; Pörtner, 1990). Low-molecular-weight organic substances that contribute to blood pigment function in vertebrates or crusta-

ceans are not found in this group. In consequence, extremely large Bohr shifts ($\Delta \log P_{50}/\Delta pH < -1$; Bridges, 1994) and very high levels of pH-dependent cooperativity are common (Miller, 1985; Pörtner, 1990). Binding of CO_2 together with O_2 in arterial blood has been suggested to support pigment function on the venous side in sepioid species (Brix *et al.*, 1981), where both O_2 and CO_2 are released, and this CO_2 helps to exploit the large Bohr effect. In squid, supplementary oxygen uptake *via* the skin supports the excessive oxygen demand and provides the excess CO_2 required for the Bohr effect to function (Pörtner, 1994).

In some cephalopods, an increase in ambient temperature has a large effect on oxygen transport by hemocyanin; this effect is reflected by an increase in cooperativity and a fall in oxygen affinity (Brix *et al.*, 1989, 1994; Mangum, 1990). If a rise in metabolic rate with temperature is supported by an adequate rise in P_{50}, the species should be able to live at a broader range of temperatures than a species in which P_{50} remains constant or in which the change in P_{50} is too large. For example, the high thermal sensitivity of the oxygen affinity of hemocyanin in the giant squid *Architeuthis monachus* suggests that arterial saturation becomes impossible at high temperatures (Brix, 1983). This question has gained general importance since comparative studies in Antarctic and temperate fish and invertebrates (including cephalopods; Pörtner and Zielinski, 1998) revealed that the limits of thermal tolerance are characterized by oxygen limitation, owing to the inability of circulation or ventilation to provide sufficient oxygen at extreme temperatures (for review, see Pörtner *et al.*, 2000). Comparison of hemocyanin oxygen binding in cephalopods of different metabolic rates and from various latitudes should show how hemocyanin oxygen transport has adapted to different temperature regimes at various levels of metabolic activity and how blood pigment function contributes to the oxygen limitation of thermal tolerance.

These questions are especially interesting for an understanding of physiological adaptations to life in Antarctica. Here the marine environment is characterized by very stable water temperatures that are close to freezing (Clarke, 1988). Under these conditions more oxygen is physically dissolved, thereby facilitating oxygen uptake and supply to tissues. At the same time metabolic rate is reduced at lower temperatures, with the consequence that in some species blood pigments may be less important (for hemocyanin, see Mauro and Mangum, 1982b; Burnett *et al.*, 1988). Some Antarctic fishes, the icefishes (Channichtyidae) have even lost their respiratory pigments (Ruud, 1954). The question arises as to whether the importance of blood pigment function is also reduced in Antarctic cephalopods.

Live specimens of the octopod *Megaleledone senoi* became available during a recent expedition to Antarctica with the RV *Polarstern*. This species is found in the indo-atlantic sector of the Antarctic Ocean (Taki, 1961; Kubodera and Okutani, 1986, 1994). In our study we investigated oxygen binding to the hemocyanin of this stenothermal Antarctic octopod by using a technique that allows continuous and simultaneous recordings of blood pH and oxygenation and the construction of diagrams depicting changes in oxygen saturation with pH (pH/saturation diagrams; Pörtner, 1990). Such an approach is most suitable for cephalopod blood owing to the extremely large pH dependence of oxygen binding (see above). It avoids the use of artificial buffers that may lead to a change in oxygen-binding properties (Pörtner, 1990; Brix *et al.*, 1994). At the same time, the amount of blood required is reduced such that more sophisticated data can be collected from the very few animals accessible in remote environments like the Antarctic. The oxygen-binding properties of *M. senoi* hemocyanin were compared with those from other eurythermal and stenothermal cephalopods. For eurythermal octopods some literature data were available. Temperature effects on oxygen binding were studied experimentally in the cuttlefish *Sepia officinalis* to complement the data set available in the literature (Lykkeboe *et al.*, 1980; Johansen *et al.*, 1982a). To some extent, cuttlefish display a mode of life similar to that of octopods. Like octopods, they live close to the bottom of the sea (von Boletzky, 1983), but they have a larger scope for activity and metabolism, which might influence the thermal adaptation of hemocyanin function.

Materials and Methods

Animals

Antarctic octopods (*Megaleledone senoi*, up to 9 kg body weight) were caught in November 1996 north of Elephant Island, Antarctica, during expedition ANT XIV/2 of the RV *Polarstern*. The animals were collected from bottom trawls. Samples were taken immediately after capture.

Cuttlefish (*Sepia officinalis*, 470 to 960 g body weight) were obtained from the Marine Biomedical Institute of the University of Texas, Galveston, Texas, where this species has been bred and grown for several consecutive generations. They were kept at a salinity of 35‰ at temperatures of 20 to 22 °C.

Sampling procedure

Animals were anesthetized by transferring them into seawater containing 2% ethanol (v/v). The animals were then removed from the seawater and the mantle was opened by a ventral incision. Blood was collected from the vena cava, the systemic heart, and the gill hearts. Blood samples from all animals were pooled, frozen, and stored for up to one year at close to −20 °C until utilized for *in vitro* studies of oxygen binding.

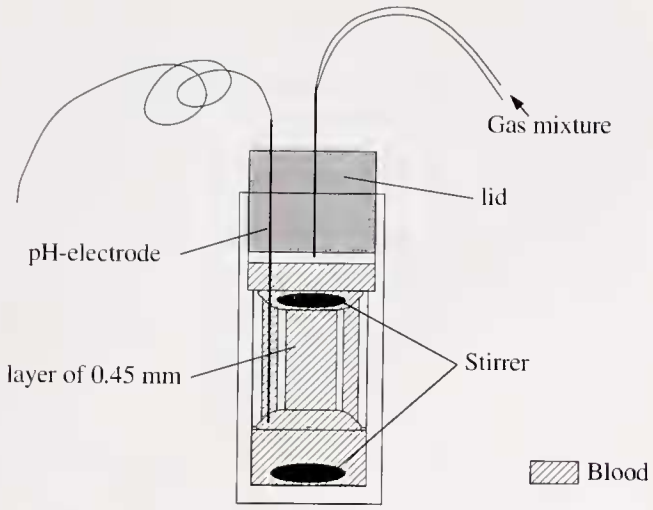

Figure 1. Cuvette used for the measurement of oxygen-binding properties. Dashed areas show the compartments of the cuvette filled with blood. Absorbance was measured through a thin layer, 0.45-mm thick, in the center part of the cuvette.

Analysis of oxygen binding

Oxygen-binding characteristics of cephalopod hemocyanin were studied using a specially constructed cuvette, built by Hellma GmbH & Co. (Mülheim, Germany; Fig. 1). The cuvette consisted of an upper and a lower compartment connected by two shafts (1.5 and 2 mm in diameter) in the left and right periphery of the cuvette, as well as a central compartment between the shafts, where blood formed a thin layer of only 0.45 mm. Stirring bars operating in the upper and lower compartments ensured continuous exchange of blood between all compartments and thus uniform mixture of the blood. Oxygen saturation was monitored continuously by using a diode array spectrophotometer with fiber optics (X-dap, IKS Optoelektronik Meßgeräte GmbH, Waldbronn, Germany) to measure absorbance at 345 nm through the thin layer. Blood samples were equilibrated by introducing humidified gas mixtures through a hole in the lid of the cuvette. Gas mixtures of variable Po_2 (between 1.0 and 20.0 kPa) were prepared from pure O_2, CO_2, and N_2 by gas-mixing pumps (type 2M303/a-F, Wösthoff, Bochum, Germany); complete deoxygenation occurred under pure N_2. Blood pH was varied by changing Pco_2 (between 0.09 and 1.01 kPa) or by replacing small volumes (<10 μl per 2 ml of blood) of supernatant plasma after ultracentrifugation (1 h at 120,000 $\times$ g; Beckman Airfuge, Beckman Instruments, Inc., Fullerton, CA) with fixed acid (1 mol l^{-1} HCl) or base (2 mol l^{-1} NaOH; Morris *et al.*, 1985; Pörtner, 1990). Changes in blood pH during oxygenation and deoxygenation of hemocyanin were measured continuously by using a needle pH electrode (long micro needle electrode #811, Diamond General Corp., Ann Arbor, MI) that was introduced into one of the shafts *via* a second hole in the lid.

Total CO_2 was analyzed at 0 °C in 50-μl blood samples of *M. senoi;* the gas chromatography method of Lenfant and Aucutt (1966) modified after Boutilier *et al.* (1985) was used. Measurements of oxygen-binding properties were carried out at 0, 5, and 10 °C for samples of *M. senoi* and at 0, 10, and 20 °C for samples of *S. officinalis;* 10 °C was chosen since this temperature can be reached in the northern part of *Sepia's* distribution range (Isemer and Hasse, 1985).

Graphical analysis and calculations

Hemocyanin concentrations were measured photometrically and calculated using the extinction coefficients of Nickerson and van Holde (1971). Oxygen capacity was estimated using the molecular weights for octopods and *Sepia* as compiled by Miller (1994) and the assumption that there are 70 O_2-binding sites per hemocyanin molecule in octopods and 80 in *Sepia.* For the evaluation of hemocyanin oxygen saturation, constant absorbance levels in the range of the highest values of Po_2 and pH were set to 100% saturation. Changes in hemocyanin oxygenation and pH were plotted in a pH/saturation diagram according to Pörtner (1990). The resulting oxygen-binding curves represent isobars delineating the change in oxygenation with pH at constant Po_2. The points of intersection of the oxygen isobars with the line of half saturation quantify P_{50}, because it depends on pH. These P_{50} and pH values were used to evaluate the Bohr coefficient, Δ log P_{50}/ΔpH by linear regression analysis. For comparison, and owing to the presence of a large pH-insensitive oxygen reserve at low temperatures, the coefficient Δ log P_{80}/ΔpH was evaluated following the same procedure. The Haldane coefficient ($\Delta HCO_3^-/\Delta HcyO_2$) was evaluated from the vertical distance between buffer lines in a pH/bicarbonate diagram (as used by Brix *et al.*, 1981). To assess whether oxygen-linked CO_2 binding to the hemocyanin occurs (Lykkeboe *et al.*, 1980), "measured" and calculated apparent bicarbonate levels were compared. Apparent "bicarbonate" (the sum of HCO_3^- and CO_3^{2-} levels) was calculated from measured CO_2 concentrations (Cco_2) using the applied Pco_2 and the measured pH according to the formula

$$[HCO_3^-] = Cco_2 - \alpha Pco_2 \qquad (1)$$

where α is the solubility of CO_2. For comparison, bicarbonate levels were also calculated according to equation (1) with Cco_2 values derived from the Henderson-Hasselbalch equation:

$$Cco_2 = Pco_2 \cdot (\alpha \cdot 10^{pH-pK'''} + \alpha) \qquad (2)$$

α and pK''' were calculated according to Heisler (1986).

Along each isobar in the pH/saturation diagram, values of saturation S depend on pH values and the Po_2 of the isobar. The pH/saturation diagram allows comparison of S and log Po_2 with P_{50} at the same pH ($=pH_{50}$). This leads to an

analysis of cooperativity at a specific pH. If this is done in the range of saturation S between 0.4 and 0.6, the analysis leads to an estimate of Hill coefficients (n_{50}) according to

$$\log(S/1 - S) = n_{50}(\log Po_2 - \log P_{50}) \qquad (3)$$

where Po_2 is the Po_2 of the isobar, S results from Po_2 at a specific pH (pH_{50}), and P_{50} is the Po_2 for $S = 0.5$ at the same pH (Pörtner, 1990).

Results

The concentration of hemocyanin in native blood (hemolymph) was 93 g l^{-1} for *Megaleledone senoi* and 142 g l^{-1} for *Sepia officinalis*. This is equivalent to a maximum level of hemocyanin-bound oxygen of 1.86 mmol O_2 l^{-1} in the octopod and of 2.84 mmol l^{-1} in *Sepia*. For *M. senoi* hemocyanin, the highest pH sensitivity of oxygen binding was found at 10 °C, as indicated by maximum slopes $\Delta S/\Delta pH$ (Fig. 2). Lower temperatures resulted in a somewhat decreased pH sensitivity of oxygen affinity, with a maximum of $\Delta S/\Delta pH = 13\%$ per pH unit at 10 °C, compared to a maximum of 10% per pH unit at 0 °C. Saturation at 0 °C did not fall below 43% even at low pH (6.4 and 6.6) and low Po_2 (1 kPa). At 10 °C, saturation dropped to a minimum of 32% at the same

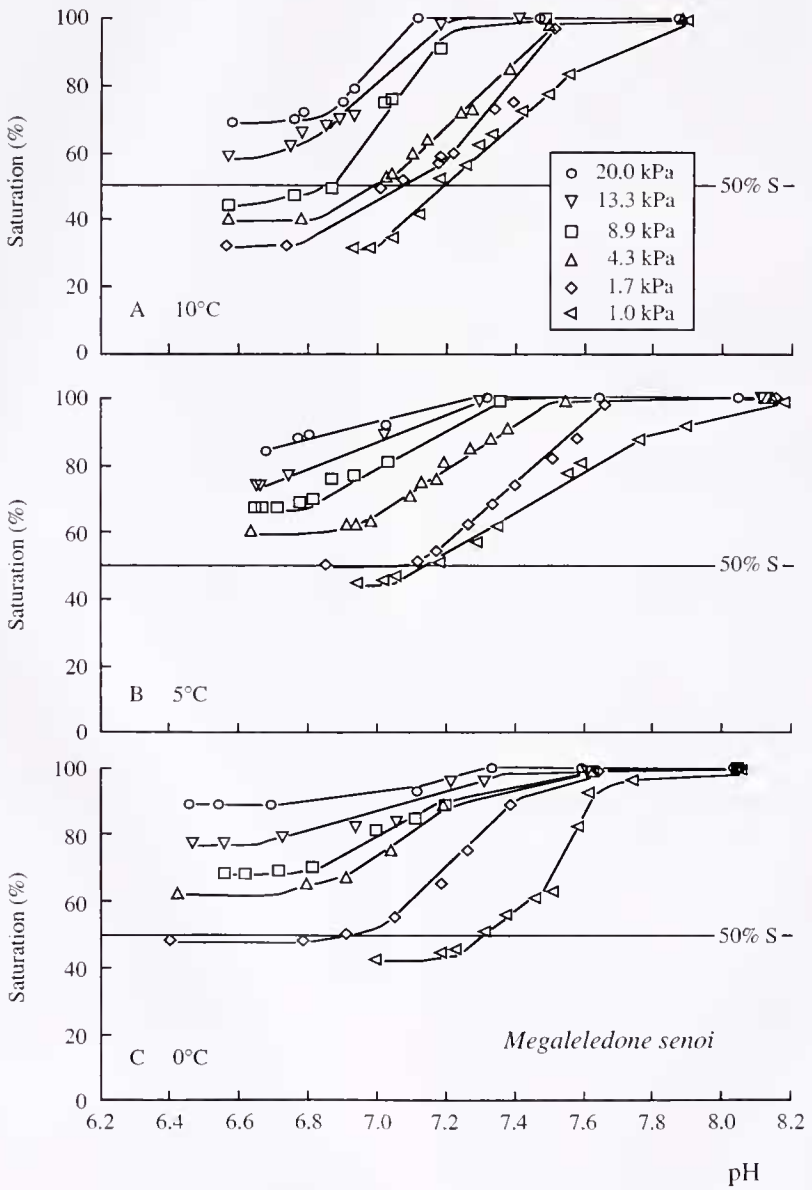

Figure 2. Continuous analysis of the relationships between oxygen binding, pH, and Po_2 in whole blood of the Antarctic octopod *Megaleledone senoi* at temperatures between 0 and 10 °C (S = saturation; points were chosen at regular intervals from the continuous recordings).

Table 1

Oxygen affinity (P_{50}), Bohr, Haldane, and Hill coefficients evaluated for the hemocyanins of the Antarctic octopod Megaleledone senoi *and of the cuttlefish* Sepia officinalis *at different temperatures*

Temperature (°C)	P_{50} (kPa)	Bohr coefficient ($\Delta \log P_{50}/\Delta$pH)	($\Delta \log P_{80}/\Delta$pH)	Haldane coefficient ($\Delta HCO_3^-/\Delta HcyO_2$)	Hill coefficient (n_{50})
Megaleledone senoi					
0	0.98 (pH 7.2)	≈ -0.9	−1.51	0.66 (pH 6.2) 0.50 (pH 6.8) 0.39 (pH 7.2)	1.4 (pH 7.43)
5		ND	−1.46	ND	1.5 (pH 7.24) 1.0 (pH 7.31)
10	1.10 (pH 7.2)	−2.33	−2.13	ND	1.2 ± 0.1 (pH 6.83–7.31; $n = 6$)
Sepia officinalis					
10	5.3 (pH 7.4)	−0.99	−1.44		4.6 (pH 7.29)
20	6.5 (pH 7.4)	−1.33	−1.94		5.9 (pH 7.48)

P_{50}, Haldane, and Hill coefficients are valid for the pH values given in brackets. ND, not determined; $HcyO_2$, concentration of oxygenated hemocyanin.

Po_2. Intermediate values of pH sensitivity and maximum unloading were found at 5 °C.

A large Bohr coefficient of −2.33 was found at 10 °C, similar to the coefficient $\Delta \log P_{80}/\Delta$ pH (Table 1). The experimental evaluation of the Bohr coefficient was not possible at lower temperatures due to the fact that pH-dependent saturation did not drop below 50% at most partial pressures of oxygen. An extrapolation of binding data to very low partial pressures of oxygen revealed a Bohr coefficient of approximately −0.9 at 0 °C, below the level of $\Delta \log P_{80}/\Delta$pH. Furthermore, oxygen affinity (P_{50}) at pH 7.2 changed only at $\Delta P_{50}/°C < 0.01$ kPa, from 0.98 kPa at 0 °C to 1.10 kPa at 10 °C (Table 1).

For *S. officinalis* hemocyanin, pH sensitivity was high at 20 °C, reaching a maximum $\Delta S/\Delta$pH of 41% per pH unit (Fig. 3). Especially in the pH range between 7.4 and 7.8, very small pH changes were sufficient to cause maximal unloading of oxygen, down to 19% saturation. The pH sensitivity at 20 °C was higher than found for *M. senoi* at all temperatures. As in *M. senoi,* lower temperatures decreased the pH sensitivity of oxygen binding with a decreased Bohr factor and level of $\Delta \log P_{80}/\Delta$pH (Table 1) and an increase in the pH-insensitive reserve at the same Po_2. At 0 °C, $\Delta S/\Delta$pH reached a maximal value of only 7% per pH unit. Oxygen saturation remained above 50% at all investigated partial pressures of oxygen and values of pH. At pH 7.4, oxygen affinity fell from $P_{50} = 5.3$ kPa at 10 °C to $P_{50} = 6.5$ kPa at 20 °C ($\Delta P_{50}/°C = 0.12$ kPa, Table 1).

The change in cooperativity with pH and temperature for *S. officinalis* is shown in Figure 4. At 20 °C, the largest Hill coefficient (n_{50}) of 5.9 was found at a pH (7.48) where pH sensitivity ($\Delta S/\Delta$pH) was also high. A decrease in temperature to 10 °C resulted in a decrease of the maximal Hill coefficient to $n_{50} = 4.6$ (pH 7.29). The maximum was shifted to lower pH. In contrast to cuttlefish, *M. sensoi* had much lower Hill coefficients (Table 1). At 0 °C, n_{50} was 1.4 (pH 7.43), and it varied between 1.0 (pH 6.83) and 1.4 (pH 7.31) at 10 °C. No clear maximum could be found.

Analysis of total CO_2 in *M. senoi* blood during variations of Po_2 and Pco_2 yields the buffer lines depicted in the pH/bicarbonate diagram (Fig. 5). The position of the buffer line shifts between oxygenated and deoxygenated blood according to the quantity of H^+ bound by the pigment. The vertical distance between the buffer lines yields the Haldane coefficient ($\Delta HCO_3^-/\Delta HcyO_2$). For *M. senoi* hemocyanin at 0 °C, the Haldane coefficient rose with falling pH (Table 1). The calculated apparent bicarbonate levels for oxygenated and deoxygenated blood diverge only slightly from the measured values, suggesting that O_2-linked CO_2 binding does not exist (Fig. 5). The non-bicarbonate buffer value (β_{NB}) of 4.25 mmol l^{-1} pH units^{-1} of *M. senoi* blood at 0 °C is in the same range as in the squids *Illex illecebrosus* and *Loligo pealei* (5.0 and 5.8 mmol l^{-1} pH units^{-1}, respectively; Pörtner, 1990).

Discussion

At the low temperatures of Antarctica icefishes rely exclusively on the transport of oxygen that is physically dissolved in the blood (see Introduction). The presence of hemocyanin-bound oxygen in *Megaleledone senoi* blood at levels similar to those seen in squids and temperate octopods (*cf.* Brix *et al.*, 1989) suggests that oxygen transport *via* hemocyanin is as important in this Antarctic species as in temperate and warm-water cephalopods. In contrast to Antarctic fishes, the unchanged requirement for blood oxygen transport in Antarctic cephalopods may be related to the

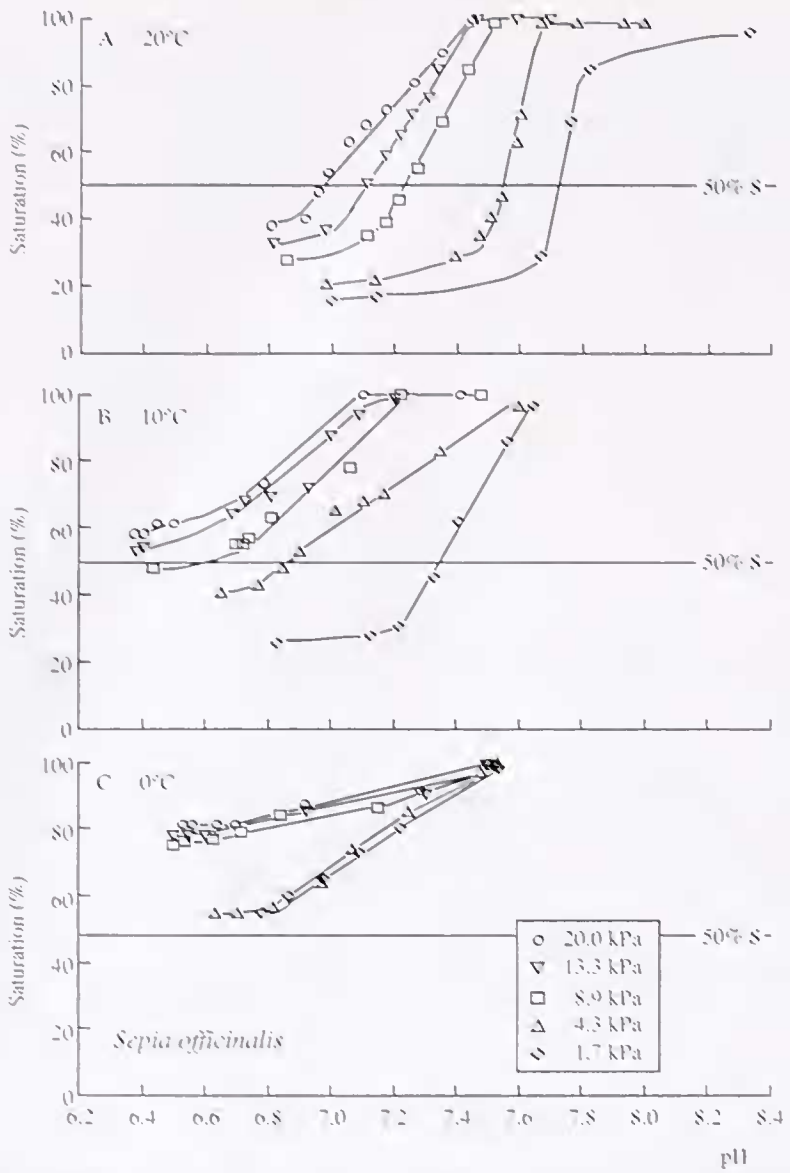

Figure 3. Depiction of oxygen-binding properties of *Sepia officinalis* hemocyanin at temperatures between 0 and 20 °C in a pH/saturation diagram. Each line indicates an oxygen isobar and shows the changes in oxygen binding depending on pH (S = saturation; points were chosen at regular intervals from the continuous recordings).

low level of capillarization of cephalopod musculature compared to fish muscles (Bone *et al.*, 1981).

In cephalopods, pH and temperature are most important factors in the regulation of hemocyanin oxygen transport (Brix *et al.*, 1989; Bridges, 1994; Pörtner, 1994). The temperature dependence of P_{50} varies greatly between species. For example, the oxygen affinity of the hemocyanin of giant squid (*Architeuthis monachus*) decreases at $\Delta P_{50}/\Delta T$ = 1.89 kPa per degree Celsius (pH 7.4), while a value of only 0.20 kPa/°C (pH 7.4) was found for the octopod *Octopus vulgaris* (calculated after Brix *et al.*, 1989). A lower value of $\Delta P_{50}/\Delta T$ = 0.10 kPa/°C (pH 7.4) was found for the octopod *Eledone cirrhosa* (calculated after Bridges, 1994); this was similar to the value of 0.12 kPa/°C (pH 7.4) calculated for *Sepia officinalis* hemocyanin in the present study. In eurythermal cephalopods like *Sepia officinalis*, *Octopus vulgaris*, or *Eledone cirrhosa* and in some squids, a moderate rise in P_{50} with temperature occurs (*cf.* Brix *et al.*, 1994). In this way capillary P_{O_2} is maintained ("buffered") at progressively higher levels, which are required for elevated diffusive oxygen flux to mitochondria during increased rates of oxygen consumption. Such changes in P_{50} with temperature allow *S. officinalis* to be distributed over a wide range, from the Mediterranean to the North Sea (von

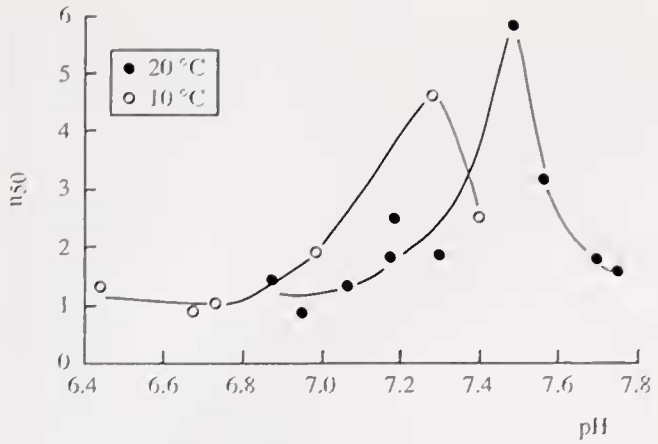

Figure 4. Hill coefficients (n_{50}) of *Sepia officinalis* hemocyanin depending on pH and temperature.

Boletzky, 1983). In contrast, the extreme thermal sensitivity of P_{50} seen in giant squid may eliminate oxygen transport by hemocyanin and contribute to the heat intolerance of these animals (Brix, 1983). The very small adjustments of P_{50} found in *M. senoi* hemocyanin ($\Delta P_{50}/\Delta T$ = 0.01 kPa/°C, pH 7.2) may also be detrimental, because when temperature rises, blood Po_2 will be held at levels too low to adequately support an increase in metabolic rate. In consequence, Antarctic octopods like *M. senoi* are very stenothermal (Pörtner and Zielinski, 1998).

For *S. officinalis* the greatest pH sensitivity (Fig. 3) at 20 °C was found in the range of *in vivo* blood pH (7.4–7.8 at 17–19 °C; Johansen *et al.*, 1982a). The same phenomenon occurs in squid (Pörtner, 1990). The fact that the oxygen transport system responds to even small changes in extracellular acid-base status is consistent with the pH-dependent Po_2 buffer function of the hemocyanin (Pörtner, 1994). This is not surprising, because cephalopods regulate primarily extracellular, not intracellular, acid-base balance (Pörtner, 1994).

The blood pH of the Antarctic octopod is not known. Assuming that blood pH follows α-stat predictions (Reeves, 1972) and is high in the cold, as seen in *Loligo pealei* blood *in vitro* (Howell and Gilbert, 1976), the *in vivo* pH range for *M. senoi* is between pH 7.7 and 7.9 at 0 °C. With the highest pH sensitivity in this pH range, oxygen unloading would occur at very low oxygen tensions (<1 kPa), supporting only very low metabolic rates (Fig. 2). A P_{50} below 1 kPa (pH 7.6; 0 °C) reflects one of the highest oxygen affinities reported so far for cephalopods. This value is close to the P_{50} of 0.47 to 0.55 kPa evaluated for the cold-water vampire squid *Vampyroteuthis infernalis* (5 °C; Seibel *et al.*, 1999). These findings suggest that *M. senoi* displays a low metabolic rate similar to that of the Antarctic octopod *Pareledone charcoti* (0.3 μmol g^{-1} h^{-1} at 0 °C and about 50 g body weight; H. O. Pörtner, T. Hirse, V. Wegewitz, unpubl. data, 15 times lower than similar sized *S. officinalis* at 17 °C, 4.4 μmol g^{-1} h^{-1}, Johansen *et al.*, 1982b) or even lower and close to the 0.1 μmol g^{-1} h^{-1} measured at 5 °C in the deep-sea squid *Vampyroteuthis infernalis* (Seibel *et al.*, 1997).

The Bohr coefficient evaluated for both investigated cephalopod species dropped when temperature decreased. This result is similar to findings in the crustaceans *Cancer magister* and *Cancer anthonyi* (Burnett *et al.*, 1988) and in the octopod *Eledone cirrhosa* (Bridges, 1994). In *S. officinalis*, $\Delta \log P_{50}/\Delta$pH decreased moderately, from −1.33 at 20 °C to −0.99 at 10 °C (Table 1). In *M. senoi*, the Bohr coefficient fell drastically, from an extremely high value of −2.33 at 10 °C to a much smaller value evaluated by extrapolation to be −0.9 at 0 °C (Table 1). The Bohr factor in the vampire squid was found to be even lower (−0.22; Seibel *et al.*, 1999). These results indicate that the Bohr effect becomes less important at low temperature and low metabolic rate.

A mechanism of oxygen-linked CO_2 binding has been proposed for *Sepia* hemocyanin, which transports both O_2 and CO_2 to the tissues. The CO_2 produced in metabolism and the CO_2 released during deoxygenation would elicit a drop in pH, as required for the large Bohr effect (< −1.0) to function normally (Lykkeboe *et al.*, 1980; Brix *et al.*, 1981). No oxygen-linked CO_2 transport was found in *M. senoi* (Fig. 5). At 0 °C, the estimated Bohr coefficient of $\Delta \log P_{50}/\Delta$pH ≈ −0.9 would reflect normal function of the Bohr effect, whereas the extremely high Bohr coefficient at 10 °C would be counterproductive for oxygen transport.

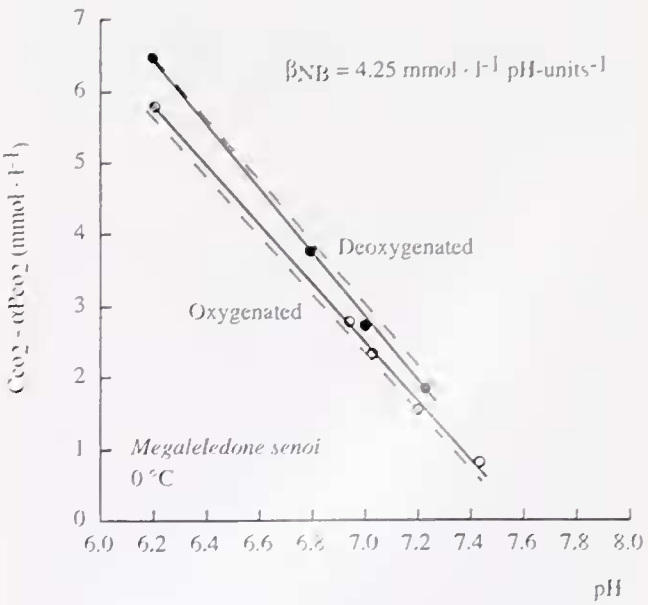

Figure 5. pH/bicarbonate diagram for whole blood of *Megaleledone senoi*. Experimental buffer lines of oxygenated and deoxygenated blood match those derived from calculated apparent bicarbonate levels (broken lines; see text).

a finding consistent with the stenothermality of Antarctic animals.

A reduced pH sensitivity of hemocyanin oxygen binding in *M. senoi* and other octopods compared to *S. officinalis* and squids is reflected in the magnitude of the pH-independent venous reserve, which rises as temperature falls (Figs. 2, 3). This reserve represents the amount of oxygen that remains bound to the respiratory pigment at constant Po_2, even when pH falls to very low values. Comparison of this venous reserve for several cephalopod species at normal environmental temperatures and low Po_2 shows that it is below 5% (at 1.7 kPa) for the squid *Illex illecebrosus* (15 °C; Pörtner, 1990), 19% (at 1.7 kPa) for *S. officinalis* (20 °C; this study), and 43% (at 1 kPa) for the Antarctic octopod *M. senoi* (0 °C; this study). A value of below 10% results for the hemocyanin of the octopod *Octopus dofleini* (at 1.7 kPa and 20 °C; Pörtner, 1990; calculated after Miller and Mangum, 1988); however, the *in vivo* value may be higher for this species because it lives at lower temperatures. The high pH sensitivity of squid hemocyanins maximizes the release of oxygen in the tissues and supports their high metabolic rate (Pörtner, 1990, 1994). Sepioids and, even more so, octopods display a less active life style with lower metabolic rates (for example: Houlihan *et al.*, 1982; Webber and O'Dor, 1985, 1986; Finke *et al.*, 1996; Seibel *et al.*, 1997). A low-activity mode of life may eliminate the necessity to maximize pH-dependent oxygen transport to the extent seen in squids.

With falling pH, the pH-independent venous reserve increased and was reached at higher Po_2 (88% at pH 6.8, 20 kPa O_2, and 0 °C in *M. senoi*, or 40% at pH 6.8, 20 kPa O_2, and 20 °C in *S. officinalis*). At normoxic Po_2 (20 kPa O_2) and low pH, this resembles a Root effect (Bridges, 1994) but at the same time, further pH sensitivity (the Bohr effect) is eliminated and deoxygenation depends exclusively on Po_2 (Figs. 2 and 3).

The cooperativity of respiratory pigments is characterized by the Hill coefficient (n_{50}). In *S. officinalis* at 20 °C (Fig. 4) and in the squids *Illex illecebrosus* and *Loligo vulgaris*, the highest cooperativity correlates with the highest pH sensitivity of oxygen binding ($\Delta S/\Delta pH$) in the range of *in vivo* pH (Pörtner, 1990). Here maximal deoxygenation occurs at minimal pH change (Pörtner, 1990, 1994). A decrease in temperature caused the maximal Hill coefficient of *S. officinalis* hemocyanin to drop from $n_{50} = 5.9$ at 20 °C to $n_{50} = 4.6$ at 10 °C. At the same time, maximum cooperativity was shifted to lower pH values, when *in vivo* pH should rise according to α-stat predictions (Reeves, 1972). A similar temperature dependence of the Hill coefficient was found for several crustaceans (Mauro and Mangum, 1982a,b). As with the Bohr effect, the progressive mismatch between the pH range of maximum cooperativity and the actual blood pH suggests that cooperativity becomes less important in oxygen transport at lower temperatures. Accordingly, a low cooperativity of $n_{50} = 1.4$ was found for *M. senoi*, at 0 °C (Table 1) and of $n_{50} = 2.2$ for the vampire squid (Seibel *et al.*, 1999). Surprisingly, cooperativity did not increase with temperature in *M. senoi* hemocyanin.

The question arises as to why thermal sensitivity is so low in *M. senoi* hemocyanin but so high in the blood pigment of cold-adapted giant squid (*cf.* Brix, 1983). A high value of ΔP_{50} °C^{-1} reflects a high heat of oxygenation (*cf.* Brix *et al.*, 1994) or Arrhenius activation energy. Giant squid probably display higher metabolic rates and thus must maintain P_{50} levels higher than those of *M. senoi*. A high heat of oxygenation may be required for setting P_{50} values high at low temperature as in giant squid. In that respect the low thermal sensitivity of hemocyanin in the Antarctic octopod is again in accordance with the low metabolic rate of this group.

In summary, the pH sensitivity of oxygen binding in cephalopod hemocyanins is adjusted to metabolic rate. The pH-insensitive oxygen reserve in hemocyanin was largest in *M. senoi* and intermediate in *S. officinalis*, if compared to squids (this study and Pörtner, 1990). Furthermore, the Bohr effect is reduced and the pH-insensitive oxygen reserve rises during cooling, suggesting that pH sensitivity falls in the cold. The temperature dependence of the Bohr factor is less pronounced in the eurythermal *S. officinalis*, which would, together with an appropriate change in P_{50}, ensure a supply of oxygen at changing temperatures. In *M. senoi*, a high oxygen affinity of hemocyanin, a moderately high Bohr coefficient, and a low cooperativity at 0 °C cause blood Po_2 to be maintained ("buffered") at low values matching a low rate of oxygen consumption. In this species the low thermal sensitivity of oxygen affinity prevents an upward shift of the buffered Po_2 at higher temperatures, suggesting that oxygen transfer to tissues may become limiting when oxygen demand rises. This observation is in contrast to the findings in giant squid, where arterial oxygen uptake is hampered by an excessive drop in oxygen affinity, thereby limiting heat tolerance (Brix, 1983). Accordingly, hemocyanin function probably contributes to an oxygen limitation of heat tolerance that sets in early and characterizes thermal tolerance in Antarctic octopods (Pörtner and Zielinski, 1998) and probably also in giant squid.

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Literature Cited

Bone, Q., A. Pulsford, and A. D. Chubb. 1981. Squid mantle muscle. *J. Mar. Biol. Assoc. UK* **61:** 327–342.

Boutilier, R. G., G. K. Iwama, T. A. Heming, and D. J. Randall. 1985. The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15 °C. *Respir. Physiol.* **61:** 237–254.

Bridges, C. R. 1994. Bohr and Root effects in cephalopod haemocyanins—paradox or pressure in *Sepia officinalis?* Pp. 121–130 in *Physiology of Cephalopod Molluscs—Lifestyle and Performance Adaptations,* H. O. Pörtner, R. K. O'Dor, and D. MacMillan, eds. Gordon and Breach, Basel.

Brix, O. 1983. Giant squids may die when exposed to warm water currents. *Nature* **303:** 422–423.

Brix, O., G. Lykkebøe, and K. Johansen. 1981. The significance of the linkage between the Bohr and Haldane effects in cephalopod bloods. *Respir. Physiol.* **44:** 177–186.

Brix, O., A. Bardgard, A. Cau, A. Colosimo, S. G. Condò, and B. Giardina. 1989. Oxygen-binding properties of cephalopod blood with special reference to environmental temperatures and ecological distribution. *J. Exp. Zool.* **252:** 34–42.

Brix, O., A. Colosimo, and B. Giardina. 1994. Temperature dependence of oxygen binding to cephalopod haemocyanins: ecological implications. Pp. 149–162 in *Physiology of Cephalopod Molluscs—Lifestyle and Performance Adaptations,* H. O. Pörtner, R. K. O'Dor, and D. MacMillan, eds. Gordon and Breach, Basel.

Burnett, L. E., D. A. Scholnick, and C. P. Mangum. 1988. Temperature sensitivity of molluscan and arthropod hemocyanins. *Biol. Bull.* **174:** 153–162.

Clarke, A. 1988. Seasonality in the Antarctic marine environment. *Comp. Biochem. Physiol.* **90B:** 461–473.

Finke, E., H. O. Pörtner, P. G. Lee, and D. M. Webber. 1996. Squid (*Lolliguncula brevis*) life in shallow waters: oxygen limitation of metabolism and swimming performance. *J. Exp. Biol.* **199:** 911–921.

Heisler, N. 1986. Buffering and transmembrane ion transfer processes. Pp. 3–47 in *Acid-Base Regulation in Animals,* N. Heisler, ed. Elsevier, Amsterdam.

Houlihan, D. G., A. J. Innes, M. J. Wells, and J. Wells. 1982. Oxygen consumption and blood gases of *Octopus vulgaris* in hypoxic conditions. *J. Comp. Physiol.* **148:** 35–40.

Howell, B. J., and D. L. Gilbert. 1976. pH-temperature dependence of the hemolymph of the squid, *Loligo pealei. Comp. Biochem. Physiol.* **55A:** 287–289.

Isemer, H. J., and L. Hasse. 1985. *The Bunker Climate Atlas of the North Atlantic Ocean. Vol I: Observations.* Springer Verlag, Berlin. 218 pp.

Johansen, K., O. Brix, and G. Lykkeboe. 1982a. Blood gas transport in the cephalopod, *Sepia officinalis. J. Exp. Biol.* **99:** 331–338.

Johansen, K., O. Brix, S. Kornerup, and G. Lykkeboe. 1982b. Factors affecting O_2 uptake in the cuttlefish, *Sepia officinalis. J. Mar. Biol. Assoc. UK* **62:** 187–191.

Kubodera, T., and T. Okutani. 1986. New and rare cephalopods from the Antarctic waters. *Mem. Natl. Inst. Polar Res., Spec. Issue* **44:** 129–143.

Kubodera, T., and T. Okutani. 1994. Eledonine octopods from the Southern Ocean: systematics and distribution. In *Southern Ocean Cephalopods: Life Cycles and Populations,* P. G. Rodhouse, U. Piatkowski and C. C. Lu, eds. *Antarct. Sci.* **6:** 205–214.

Lenfant, C., and C. Aucutt. 1966. Measurement of blood gases by gas chromatography. *Respir. Physiol.* **1:** 398–407.

Lykkeboe, G., O. Brix, and K. Johansen. 1980. Oxygen-linked CO_2 binding independent of pH in cephalopod blood. *Nature* **287:** 330–331.

Mangum, C. P. 1983. Oxygen transport in the blood. Pp. 373–429 in *The Biology of Crustacea,* Vol. 3, L. H. Mantel, ed. Academic Press, New York.

Mangum, C. P. 1990. Gas transport in the blood. Pp. 443–468 in *Squid as Experimental Animals,* D. L. Gilbert, E. J. Adelman, Jr., and J. M. Arnold, eds. Plenum, New York.

Mauro, N. A., and C. P. Mangum. 1982a. The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. I. Intraspecific responses to seasonal differences in temperature. *J. Exp. Zool.* **219:** 179–188.

Mauro, N. A., and C. P. Mangum. 1982b. The role of the blood in the temperature dependence of oxidative metabolism in decapod crustaceans. II. Interspecific adaptations to latitudinal changes. *J. Exp. Zool.* **219:** 189–195.

Miller, K. I. 1985. Oxygen equilibria of *Octopus dofleini* hemocyanin. *Biochemistry* **24:** 4582–4586.

Miller, K. I. 1994. Cephalopod hemocyanins. A review of structure and function. Pp. 101–120 in *Physiology of Cephalopod Molluscs—Lifestyle and Performance Adaptations,* H. O. Pörtner, R. K. O'Dor, and D. MacMillan, eds. Gordon and Breach, Basel.

Miller, K., and C. P. Mangum. 1988. An investigation of the nature of Bohr, Root, and Haldane effects in *Octopus dofleini* hemocyanin. *J. Comp. Physiol.* **158B:** 547–552.

Morris, S., A. C. Taylor, C. R. Bridges, and M. K. Grieshaber. 1985. Respiratory properties of the haemolymph of the intertidal prawn *Palaemon elegans* (Rathke). *J. Exp. Zool.* **233:** 175–186.

Nickerson, K. W., and K. E. van Holde. 1971. A comparison of molluscan and arthropod hemocyanin. I. Circular dichroism and absorption spectra. *Comp. Biochem. Physiol.* **39B:** 855–872.

O'Dor, R. K., and D. M. Webber. 1986. The constraints on cephalopods: why squid aren't fish. *Can. J. Zool.* **64:** 1591–1605.

Pörtner, H. O. 1990. An analysis of the effects of pH on oxygen binding by squid (*Illex illecebrosus, Loligo pealei*) haemocyanin. *J. Exp. Biol.* **150:** 407–424.

Pörtner, H. O. 1994. Coordination of metabolism, acid-base regulation and haemocyanin function in cephalopods. Pp. 131–148 in *Physiology of Cephalopod Molluscs—Lifestyle and Performance Adaptations,* H. O. Pörtner, R. K. O'Dor, and D. MacMillan, eds. Gordon and Breach, Basel.

Pörtner, H. O., and S. Zielinski. 1998. Environmental constraints and the physiology of performance in squids. In *Cephalopod Biodiversity, Ecology and Evolution,* A. I. L. Payne, M. R. Lipinski, M. R. Clarke, and M. A. C. Roeleveld, eds. *S. Afr. J. Mar. Sci.* **20:** 207–221.

Pörtner, H. O., P. L. M. van Dijk, I. Hardewig, and A. Sommer. 2000. Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. Pp. 109–122 in *Antarctic Ecosystems: Models for Wider Ecological Understanding.* W. Davison and C. Howard Williams, eds. Caxton Press, Christchurch, New Zealand.

Reeves, R. B. 1972. An imidazole alphastat hypothesis for vertebrate acid-base-regulation: tissue carbon dioxide content and body temperature in bullfrogs. *Respir. Physiol.* **81:** 255–274.

Roper, C. F. E., M. J. Sweeney, and C. E. Nauen. 1984. *FAO Species Catalogue. Vol. 3: Cephalopods of the World. An Annotated and Illustrated Catalogue of Species of Interest to Fisheries. FAO Fisheries Synopses 125,* FAO/UNDP, Rome. 277 pp.

Ruud, J. T. 1954. Vertebrates without erythrocytes and blood pigment. *Nature* **173:** 848–850.

Seibel, B. A., E. V. Thuesen, J. J. Childress, and L. A. Gorodezky. 1997. Decline in pelagic cephalopod metabolism with habitat depth reflects differences in locomotory efficiency. *Biol. Bull.* **192:** 262–278.

Seibel, B. A., F. Chausson, F. H. Lallier, F. Zal, and J. J. Childress. 1999. Vampire blood: respiratory physiology of the vampire squid (Cephalopoda: Vampyromorpha) in relation to the oxygen minimum layer. *Exp. Biol. Online* **4:** 1.

Senozan, N. M., A. Avinc, and Z. Unver. 1988. Hemocyanin levels in *Octopus vulgaris* and the cuttlefish *Sepia officinalis* from the Aegean sea. *Comp. Biochem. Physiol.* **91A:** 581–585.

Shadwick, R. E., R. K. O'Dor, and J. M. Gosline. 1990. Respiratory and cardiac function during exercise in squid. *Can. J. Zool.* **68:** 192–198.

Urich, K. 1990. *Vergleichende Biochemie der Tiere.* Gustav Fischer, Stuttgart. 708 pp.

Taki, I. 1961. On two new eledonid octopods from the Antarctic sea. *J. Fac. Fish. Anim. Husb. Hiroshima Univ.* **3:** 297–316.

von Boletzky, S. 1983. *Sepia officinalis.* Pp. 31–52 in *Cephalopod Life Cycles,* Vol. I. P. R. Boyle, ed. Academic Press, London.

Webber, D. M., and R. K. O'Dor. 1985. Respiration and swimming performance of short-finned squid (*Illex illecebrosus*). *Northwest Atl. Fish. Organ. Sci. Counc. Stud.* **9:** 133–138.

Webber, D. M., and R. K. O'Dor. 1986. Monitoring the metabolic rate and activity of free-swimming squid with telemetered jet pressure. *J. Exp. Biol.* **126:** 203–224.

Wells, M. J., R. T. Hanlon, P. G. Lee, and F. P. Dimarco. 1988. Respiratory and cardiac performance in *Lolliguncula brevis* (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. *J. Exp. Biol.* **138:** 17–36.